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Inhalation of Steady-State Sidestream Smoke From One Cigarette Promotes Arteriosclerotic Plaque Development

Arthur Penn, PhD; Lung-Chi Chen, PhD; Carroll A. Snyder, PhD

Background A number of epidemiologic studies have suggested that every year environmental tobacco smoke (second-hand smoke) is responsible for tens of thousands of deaths, mostly from heart disease, in the United States. Environmental tobacco smoke is composed mainly (80% to 85%) of aged and diluted sidestream smoke. The remainder is exhaled mainstream smoke. Among the thousands of compounds that have been identified in environmental tobacco smoke are a number of carcinogens, including polynuclear aromatic hydrocarbon carcinogens, such as benzo(a)pyrene. We have demonstrated previously that a number of carcinogens, including benzo(a)pyrene, promote plaque development after injection into cockerels. There have been almost no studies showing a direct stimulatory effect of environmental tobacco smoke on plaque development. Recently we demonstrated that cockerels exposed to sidestream smoke for approximately 0.4% of their projected lifespan exhibited accelerated development of arteriosclerotic plaques.⁶ In that study, cockerels in specially designed inhalation chambers were exposed to the steady-state sidestream smoke from 5 cigarettes for 6 h/d for 16 weeks. This level of exposure is high but environmentally plausible. Statis-

tically significant increases in plaque size were demonstrated in the smoke-exposed cockerels.

Methods and Results In the present study, exposure levels were decreased by a factor of 5. Thirty cockerels were exposed to the steady-state sidestream smoke from 1 cigarette for 6 hours per day for 16 weeks. The smoke was mixed with filtered air. Ten control cockerels were exposed to filtered air only. Levels of smoke surrogates, including carbon monoxide and total suspended particulates, were measured three times a day. Again, there was a statistically significant increase in plaque size in the smoke-exposed cockerels. To place these studies within a context of environmental relevance, levels of carbon monoxide were measured independently over 1 to 3 hours in four bars where there was heavy smoking. Measured carbon monoxide levels were as high or higher in the bars than they were in the exposure chambers during the 1-cigarette sidestream-smoke study.

Conclusions Experimental exposure to secondhand smoke at levels equal to or even below those routinely encountered by people in smoke-filled environments is sufficient to promote arteriosclerotic plaque development. (*Circulation*. 1994;90:1363-1367.)

Key Words • arteriosclerosis • smoking

During the past decade there has been a growing public perception that involuntary exposure to environmental tobacco smoke ("second-hand smoke") can result in an increased, if poorly defined, health hazard. Numerous governmental offices as well as private establishments have severely restricted the areas where smoking is still permitted or have banned smoking entirely. Epidemiologic data have lent support to the public perceptions of the dangers posed by environmental tobacco smoke. The American Heart Association in a recent position paper recommended that environmental tobacco smoke be classified as an environmental poison and described it as a major preventable cause of cardiovascular disease.¹ Epidemiologic studies have attributed thousands of excess heart disease deaths yearly to involuntary exposure to environmental tobacco smoke.²⁻⁴ However, unlike lung cancer, where pathophysiological alterations resulting from voluntary inhalation of mainstream cigarette smoke have been well documented, studies in which cardiovas-

cular pathology has been related directly to environmental tobacco smoke exposures are very rare.⁵ Recently, we demonstrated that inhalation of sidestream cigarette smoke, a surrogate for environmental tobacco smoke, markedly accelerates development of arteriosclerotic plaques in an experimental animal model.⁶

In cockerels, abdominal aorta arteriosclerotic plaques arise spontaneously.⁷ These plaques are similar histologically and ultrastructurally to human coronary artery plaques. Normally, these plaques develop slowly and are microscopic during the first year of life. However, a variety of environmental agents can accelerate development of these plaques to the point where some are visible to the naked eye in cockerels killed at 6 months of age.⁸⁻¹⁰ In our first series of secondhand smoke studies, cockerels were exposed in dynamic exposure chambers and inhaled sidestream smoke generated by the steady-state combustion of 5 moderate-tar reference cigarettes.⁶ Exposures lasted 6 hours per day, 5 days per week, for 16 weeks. There were no differences in incidence or anatomic distribution of plaques between sidestream smoke-exposed and air-exposed control cockerels. However, plaque sizes were significantly larger in the sidestream smoke-exposed group. Although the exposures were relatively brief (approximately 0.4% of projected life span), the levels of smoke generated were higher than would be encountered in many environmental situations.

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From the Nelson Institute of Environmental Medicine, New York University Medical Center, Tuxedo, NY.

Correspondence to Arthur Penn, PhD, Nelson Institute of Environmental Medicine, New York University Medical Center, Long Meadow Rd, Tuxedo, NY 10987.

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Reduced exposure
What is the significance
of this study now??

Because of the difficulty of reliably estimating the numbers of cigarettes that contribute to environmental tobacco smoke in restaurants, bars, etc, three surrogates—carbon monoxide, total suspended particulates, and nicotine—are measured routinely to provide estimates of the amount of sidestream smoke that is generated. The published values for these surrogates in public venues vary by up to two orders of magnitude.¹¹ In our initial study, relatively high steady-state levels of these surrogates were measured in the smoke exposure chambers. The relevance of these levels to those reportedly encountered in environmental situations is the subject of some debate.^{12,13}

In the present study, we lowered the smoke exposures by a factor of 5. Cockerels were exposed to the sidestream smoke from the steady-state combustion of 1 cigarette. The timing and duration of exposure were the same as in the first series of studies. Plaque sizes were again significantly larger in the sidestream smoke-exposed group than in controls, although the magnitude of the size increase was lower than in the 5-cigarette study. The environmental relevance of this study is highlighted by independent measurements of carbon monoxide levels that were made in commercial establishments where cigarette smoking was taking place. In four of four cases, steady-state carbon monoxide levels equaled or exceeded the carbon monoxide levels measured in the smoke exposure chambers during the 1-cigarette study.

Methods

The general methods and experimental approaches have been described.⁶ Here we will summarize those methods and approaches and describe in detail only those methods that differ significantly from those we reported previously.

Animals

Four-week-old white leghorn cockerels (Avian Services) were quarantined for 2 weeks before being randomly distributed into sidestream smoke-exposed and air-exposed control groups. They were adapted to a 12-h light/12-h dark cycle, and their health and behavior were monitored constantly. AALAC guidelines were followed throughout for housing and care.

Exposures

Simultaneous exposures were carried out in the same stainless steel dynamic inhalation chambers¹⁴ used previously.⁶ Either 6 or 8 cockerels were exposed to sidestream smoke in each of four chambers. Ten control cockerels were exposed to filtered air in identical chambers.

Sidestream smoke was generated by a commercial smoking machine (AMESA Technologies) that we modified to smoke 1 cigarette at a time. The sidestream smoke was mixed with HEPA-filtered air and sent to each of the four exposure chambers. Airflow was maintained to each chamber at 300 L/min (ie, about 14 air changes per hour). Temperature in each chamber was kept at $70 \pm 1^\circ\text{C}$, while relative humidity was ambient. The same puff characteristics (30-mL puff, 2-second duration, 15-second intervals), ejection, and cigarette replacement procedures were used as described previously.⁶

The three smoke surrogates were measured routinely to provide independent assessments of the amount of sidestream smoke present in each chamber at a steady state. Carbon monoxide and total suspended particulates were measured three times per day, while nicotine levels were measured weekly as described previously.⁶

Exposure Protocols

Thirty white leghorn cockerels were exposed for 6 hours per day, 5 days per week, from 6 to 22 weeks of age to sidestream smoke produced by the steady-state combustion of one 1R4F moderate-tar reference cigarette (THRI, University of Kentucky, Lexington). Ten control cockerels in adjacent chambers were exposed simultaneously to filtered conditioned air.

Plaque Analysis

After the cockerels were killed, aortas were removed, fixed, coded, and processed as described previously.⁶ Cross sections 50- μm thick were cut from the distal face of each of 10 consecutive abdominal aorta segments starting at the iliac bifurcation (segment 1) and moving rostrally to the start of the thoracic aorta (segment 10). Sections were stained with Verhoeff's and van Gieson's stain, and plaque size was determined by computerized morphometry. Plaque size was expressed as the plaque index (mean plaque cross-sectional area [square millimeters]/mean luminal circumference [millimeters] $\times 100$). Means were determined in triplicate for each slide from each cockerel. All slides were read double blind.

Data Analysis

Before beginning this experiment we carried out a power study based on the findings in the 5-cigarette sidestream-smoke study. The results indicated that if inhalation of increasing amounts of sidestream smoke resulted in a linear increase in plaque size, then in the proposed experiments 30 smoke-exposed and 10 control cockerels should suffice to yield statistically significant differences in plaque size between the two groups.

As reported in the 5-cigarette study, the measured plaque index values fitted log-normal distributions.⁶ The logarithms of smoke-exposed and air-exposed control plaque index values were arranged according to increasing value and plotted on log-probability coordinates. Linear regression lines were calculated via least-squares analysis and drawn for each data set. ANCOVA was used to test for differences between the two regression lines.

Environmental Carbon Monoxide Measurements

To compare carbon monoxide levels in the smoking chambers with those encountered in "real world" situations, we used a calibrated, portable monitor (National Draeger Model 190/DL/CO) to measure carbon monoxide levels in two bars and two bar/restaurants on crowded evenings. Measurements were taken continuously (1 to 3 hours) while smoking occurred in each establishment.

Results

Steady-state exposure chamber concentrations (mean \pm SEM for each of four chambers) of carbon monoxide, total suspended particulates, and nicotine are presented in Table 1. Carbon monoxide levels averaged 4.33 ppm in the four chambers, suspended particulates averaged 2.48 mg/m^3 , and nicotine values averaged 108.3 $\mu\text{g}/\text{m}^3$. Nicotine levels were higher in chambers D and E than in chambers A and B. We have no explanation for these differences, which had no discernable effect on the results reported below.

Plaque analysis data are presented in Table 2. Microscopic plaques were present in the abdominal aortas of all cockerels in each group. Neither the number of plaques per cockerel nor the mean number of plaque-containing aortic segments per cockerel was significantly different in the two groups (Table 2, Student's *t* test, $P > .10$ in both cases). There were 63 of 100 (63%) plaque-containing abdominal aorta segments from control cockerels and 200 of 295 (68%) plaque-containing aortic segments from sidestream smoke-exposed cock-

TABLE 1. Average Daily Concentrations of Smoke Surrogates During 16-Week Exposure to Sidestream Smoke From One Cigarette

	Chamber A	Chamber B	Chamber D	Chamber E
Total suspended particulates, mg/m ³	2.76±0.08	2.51±0.08	2.43±0.07	2.21±0.06
CO, ppm	4.50±0.13	4.49±0.13	4.17±0.09	4.15±0.09
Nicotine, µg/m ³	86.7±13.7	92.5±2.4	125.5±13.9	128.4±18.4

Values are mean±SEM. Smoke exposures were carried out simultaneously in four identical 1.3-m³ chambers. Total suspended particulates and CO were measured three times a day. Nicotine was measured weekly (n=15 for chamber A and n=16 for chambers B, D, and E).

erels. Five segments from one aorta in the latter group could not be processed. These frequencies are not significantly different (χ^2 test, $P>.25$). In addition, the distribution of plaque along the length of the abdominal aorta was very similar in both groups (Fig 1).

Although inhalation of the steady-state sidestream smoke from 1 cigarette had no effect on plaque numbers or on their spatial distribution, it did result in a measurable increase in plaque sizes. The average plaque index values on a segment-by-segment basis for all cockerels in both groups are presented in Fig 2. For each of the 10 sets of segments (Nos. 1 through 10) in the 10 controls, 3 to 8 samples had plaques. For each of the 10 sets of segments in the 30 smoke-exposed cockerels, 12 to 26 segments had plaques. Except for the No. 1 segments, mean plaque index values (solid line) were larger for smoke-exposed (Fig 2A) than for control cockerels (Fig 2B). In both smoke-exposed and air-exposed control cockerels, mean plaque index values (solid lines) were greater than corresponding median values (dashed lines) for 8 of 10 aortic locations. To carry out appropriate statistical analysis, these skewed plaque index values were transformed. Both sets of data fit log-normal distributions. This was consistent with previous findings of plaque size distributions in cockerels.^{8,9,15} The logarithms of the plaque indices from 63 control and 200 smoke-exposed samples were ranked by increasing value and plotted on log-probability coordinates (Fig 3). Linear regression lines calculated via least-squares analysis were drawn for each data set. The linear correlation coefficients for each regression line exceeded 0.975. The two linear regression lines were tested for differences by ANCOVA. Plaque sizes were significantly larger in the smoke-exposed cockerels ($F=75.8$; $P<.0001$). The two regression lines intersect at a probit value of 4.05. This corresponds to a plaque index value of 0.76 (log plaque index, -0.12). The region of overlap covers the lowest 18% of plaque index values in both groups. Above this value the two popu-

lations diverge. These results indicate that inhalation of steady-state sidestream smoke from as little as 1 cigarette by young animals, for as little as 0.4% of their projected lifespan,¹⁶ is sufficient to exacerbate arteriosclerotic plaque development.

Finally, we sought an objective assessment of the relation of exposure-chamber smoke surrogate measurements to the doses of environmental tobacco smoke to which people can be exposed routinely. We measured carbon monoxide levels for periods ranging from 60 to 180 minutes in a series of establishments where cigarettes were being smoked. Data are presented in Table 3. When smokers were more than 12 ft from the portable carbon monoxide monitor in an air-conditioned bar, the monitor registered 1 ppm. When four people smoked within 8 ft of the monitor in the same room, 5 to 6 ppm were registered. After smoking stopped, the carbon monoxide level returned to 1 ppm within 30 seconds. In a second room, two active smokers within 5 ft of the monitor kept carbon monoxide levels at 3 to 4 ppm. With one smoker, 2 ppm were registered. Four smokers 25 ft from the monitor had no measured effect. In a third case, at a crowded bar, continuous measurements were taken over a 3-hour period. There were 8 to 10 smokers within 10 ft of the monitor during this time. The 3-hour time-weighted average carbon monoxide concentration was 8 ppm, and the peak concentration was 20 ppm. In a fourth establishment with steady-state smoke from 8 to 10 cigarettes within 8 ft of the monitor, the 1-hour time-weighted average carbon monoxide concentration was 5 ppm, with a peak

TABLE 2. Number of Plaques and Plaque-Containing Segments per Cockerel

	Air-Exposed Controls (n=10)	Sidestream Smoke-Exposed (n=30)
Plaques per cockerel	1.90±0.24*	1.63±0.14*
Plaque-containing segments per cockerel	6.30±0.80†	6.67±0.48†

*These values are not significantly different (Student's *t* test; with *df*=38 and *t*=1.28, $P>.10$).

†These values are not significantly different (Student's *t* test; with *df*=38 and *t*=0.39, $P>.10$).

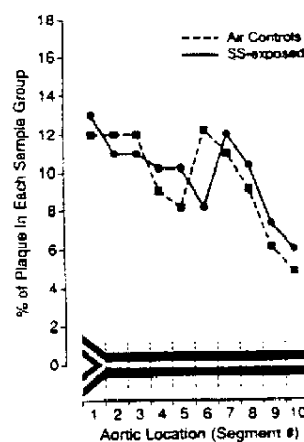


Fig 1. Plot showing percentage of plaque in sidestream smoke (SS)-exposed (—) and air-exposed control (---) cockerels versus aortic location.

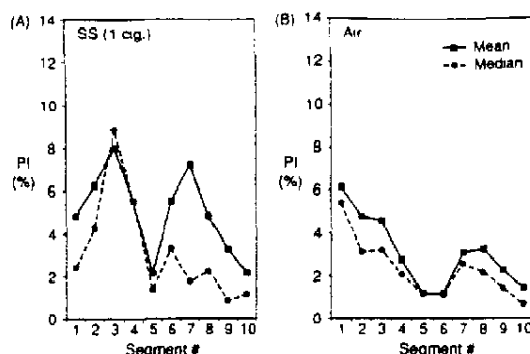


Fig 2. Plots showing comparison of mean (—) and median (---) plaque index (PI) values per segment for sidestream smoke (SS)-exposed (A) and air-exposed control (B) cockerels. Note that mean values exceed corresponding medians in 16 of 20 cases.

value of 13 ppm. In all cases the kitchens in these establishments were more than 12 ft from the monitor, so incomplete combustion of cooking gases cannot explain the recorded carbon monoxide levels. Thus, people in enclosed public areas where secondhand cigarette smoke is being generated can be exposed to carbon monoxide levels that equal or exceed the levels we measured in the cockerel exposure chambers during the present secondhand smoke study.

Discussion

Whether inhalation of environmental tobacco smoke results directly in quantifiable, deleterious health effects has been the subject of some controversy. Most of the studies purporting to show such a relation have been retrospective epidemiology studies, which depend heavily on the memory of surviving, nonsmoking spouses and other relatives of deceased smokers. These data were deemed sufficiently compelling regarding lung cancer that the Environmental Protection Agency classified sidestream smoke as a human carcinogen.¹⁷ About 3000

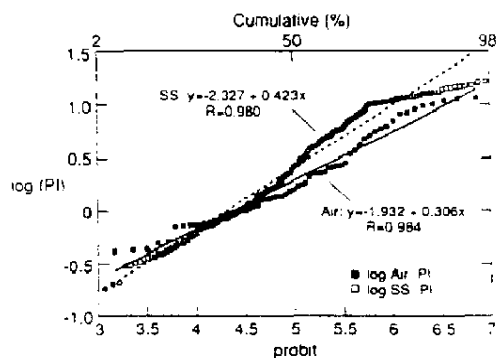


Fig 3. Plot showing log-normal distribution of plaque sizes. Plaque indexes (PI) are plotted versus probit units (lower abscissa) and cumulative percent of plaque-containing segments (upper abscissa) on log-probability coordinates. Linear regression lines calculated via least-squares analysis were drawn for each data set. Two hundred plaque index values are represented on the upper line (sidestream smoke (SS)) and 63 on the lower line (air control). A probit value of 5 corresponds to the geometric mean of each log-normal distribution. The two lines intersect at a probit value of 4.05. The region of overlap covers the lowest 18% of plaque index values in the two groups.

TABLE 3. Carbon Monoxide Levels Associated With Environmental Smoke in Four Bars/Restaurants

	1	2	3	4
No. of smokers	4*	1-2†	8-10‡	8-10‡
Measurement period, min	120†	60	180	60
CO, average ppm	6	2-4	8	5
CO, peak ppm			20	13

*Intermittent smoking over 120 minutes. CO values dropped to 1 ppm within 30 seconds of cessation of smoking.

†At least 1 cigarette was being smoked at all times during the 60 minutes when measurements were made.

‡At least 8 cigarettes were being smoked at all times when measurements were made. When smokers were > 12 ft from the monitor, CO levels registered 1 ppm.

lung cancer deaths yearly among US nonsmokers were attributed to inhalation of secondhand smoke. Recently, the appearance of precancerous lesions has been associated with exposure to environmental tobacco smoke.¹⁸ Estimates of excess yearly heart disease deaths in the United States due to exposure to secondhand smoke are an order of magnitude higher than the estimates of excess lung cancer deaths.²⁻⁴ However, with the exception of one recent study showing that arteriosclerosis was exacerbated in rabbits on a high-cholesterol diet who were exposed to fairly high levels of secondhand smoke,⁹ ours have been the only reported systematic studies on direct cardiovascular system effects due to exposure to secondhand smoke. Part of the reason for this is technical. Relatively few independent research institutions have the appropriate facilities for carrying out well-controlled exposure studies with secondhand smoke. The studies described here and in the previous 5-cigarette study⁶ were carried out at the Inhalation Exposure Facility at New York University Medical Center. In the earlier double-blind study, cockerels exposed to the steady-state sidestream smoke from 5 filtered cigarettes, for 6 hours per day for 80 days, displayed significant increases in arteriosclerotic plaque size compared with values from controls exposed to filtered air. However, the steady-state levels of smoke surrogates that we measured in the exposure chambers were higher than most published values for smoke surrogates in the environment. In the study reported here we lowered the number of cigarettes smoked by a factor of 5. Compared with our previous study using sidestream smoke from 5 cigarettes, suspended particulates and nicotine in the 1-cigarette study were lower by a factor of approximately 3, and carbon monoxide levels were lower by a factor of approximately 8. In addition, we carried out a set of field measurements of carbon monoxide in establishments where secondhand smoke was being generated. The results of the cockerel study show that inhalation of the steady-state sidestream smoke from even 1 cigarette results in significant acceleration of plaque development. An additional finding was that nonsmokers in smoky environments are routinely exposed to levels of carbon monoxide that equal or exceed the levels we found in sidestream smoke that promoted arteriosclerosis in test animals.

The cockerel is a particularly appropriate model for plaque acceleration studies. It is well established that the fibromuscular abdominal aorta plaques in the cock-

erel are very similar histologically and ultrastructurally to fibrous coronary artery plaques in humans.⁷ The standard chow diet fed to the cockerels is low in cholesterol and saturated fat. The plaques that appear in the abdominal aorta have little lipid involvement. However, if the diets are supplemented with 1% cholesterol, plaque sizes increase by more than a factor of 2.¹⁹ Whether these animals ingest cholesterol as a diet supplement, receive carcinogens by injection,^{8,9,15} or inhale cigarette smoke,^{6,20} the result is the same: accelerated development of arteriosclerotic plaques. The effect of these exposures is to increase the size of microscopic plaques to the point where many become visible to the naked eye. There are no significant increases in the actual numbers of plaques and no differences in areas within the aorta where plaques originate compared with control values.

These findings may have important public health implications. The unexpectedly high levels of coronary heart disease found at autopsy of US soldiers killed during the Korean War indicated that arteriosclerotic disease begins in the very young.²¹ These results have been confirmed and expanded in both the PDAY and Bogalusa Heart studies (eg, see References 22 and 23). Of the exogenous agents that can modulate plaque development, the most prominent ones and also the ones most amenable to change are dietary factors and cigarette smoke. There are many reports of regression of diet-associated atherosclerosis in animals, including regression of early-stage lesions.²⁴⁻²⁶ There are also at least two reports of drug treatments that resulted in regression of advanced human coronary artery disease.^{27,28} Mainstream smoke data for both cancer and heart disease indicate that as the time lapse since cessation of smoking increases, life expectancy for ex-smokers approaches that of people who never smoked.²⁹ These findings imply that smoking acts primarily to promote development of arteriosclerotic plaques that already exist. The results here combined with those from the previous 5-cigarette study support this view and demonstrate that involuntary exposure to secondhand smoke at levels equal to or even lower than those found in routinely encountered smoking environments will accelerate arteriosclerotic plaque development. The effect may be especially pronounced, and largely avoidable, in young children. We have not determined yet whether the smoke-associated plaque size increases in cockerels are reversible.

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